Biochemical and Biophysical Research Communications 451 (2014) 288–294

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Prolyl-hydroxylase PHD3 interacts with pyruvate dehydrogenase (PDH)-E1β and regulates the cellular PDH activity

Daisuke Kikuchi [a], Yoji Andrew Minamishima [b,c], Koh Nakayama [a,*]

[a] Oxygen Biology Laboratory, Medical Research Institute, Tokyo Medical and Dental University, Bunkyo-ku, Tokyo 113-8510, Japan
[b] Department of Biochemistry, School of Medicine, Keio University, Shinjuku-ku, Tokyo 160-8582, Japan
[c] Japan Science and Technology Agency (JST), Exploratory Research for Advanced Technology (ERATO), Suematsu Gas Biology Project, Shinjuku-ku, Tokyo 160-8582, Japan

ARTICLE INFO

Article history:
Received 23 July 2014
Available online 1 August 2014

Keywords:
Prolyl-hydroxylase
PHD3
Pyruvate dehydrogenase complex
PDH-E1β
Hypoxia

ABSTRACT

Cells are frequently exposed to hypoxia in physiological and pathophysiological conditions in organisms. Control of energy metabolism is one of the critical functions of the hypoxic response. Hypoxia-Inducible Factor (HIF) is a central transcription factor that regulates the hypoxic response. HIF prolyl-hydroxylase PHDs are the enzymes that hydroxylate the α subunit of HIF and negatively regulate its expression. To further understand the physiological role of PHD3, proteomics were used to identify PHD3-interacting proteins, and pyruvate dehydrogenase (PDH)-E1β was identified as such a protein. PDH catalyzes the conversion of pyruvate to acetyl-coA, thus playing a key role in cellular energy metabolism.

PDH activity was significantly decreased in PHD3-depleted MCF7 breast cancer cells and *PHD3*$^{-/-}$ MEFs. PHD3 depletion did not affect the expression of the PDH-E1α, E1β, and E2 subunits, or the phosphorylation status of E1α, but destabilized the PDH complex (PDC), resulting in less functional PDC. Finally, *PHD3*$^{-/-}$ cells were resistant to cell death in prolonged hypoxia with decreased production of ROS.

Taken together, the study reveals that PHD3 regulates PDH activity in cells by physically interacting with PDC.

1. Introduction

Cells constantly consume oxygen and utilize it for efficient energy production. When there is an imbalance between the oxygen demand and supply, which is caused by inappropriate cell growth, defective blood flow, or decreased oxygen in the surrounding environment, cells promptly adapt to such condition by hypoxic responses. Hypoxia-Inducible Factor (HIF) is a transcription factor that plays a central role in the hypoxic response [1]. HIF induces a number of its target genes to regulate the physiological response to hypoxia.

The α subunit of HIF (HIF-α) is down-regulated in normoxia and stabilized in hypoxia [1]. The HIF prolyl-hydroxylase PHDs are key molecules that regulate the oxygen-dependent expression of HIF-α. PHDs hydroxylate prolyl residues in HIF-α, thereby inducing the interaction between HIF-α and the ubiquitin ligase pVHL and leading HIF-α to degradation [2]. There are three major PHDs in mammals, PHD1, PHD2 and PHD3, and a recently characterized PHD4 [3,4]. PHD2 is the main enzyme that hydroxylates HIF-α in normoxia [5]. PHD3 also functions during chronic hypoxia or upon reoxygenation [6]. There are reports showing that PHDs have substrates other than HIF-α, such as Rpb1, IKK-β, or PKM2, indicating that PHDs are also involved in other cellular signaling pathways [7–9].

Previously, we reported that PHD3 forms a large complex during hypoxia to inhibit its ability to hydroxylate HIF-α [10]. To further characterize the complex, we performed a proteomic analysis to search for PHD3-interacting proteins and identified several candidates. PRP19 was one of such proteins which interacts with PHD3 and regulates cell death in hypoxia [11]. PDH-E1β was another candidate protein identified in the same proteomic analysis. To further understand the role of the PHD3 complex in cells, we characterized PDH-E1β in the present study.

PDH is the mitochondrial enzyme that catalyzes the conversion of pyruvate into acetyl co-A, thus connecting glycolysis and the tricarboxylic acid cycle. PDH functions as a large complex consisting of four main subunits: E1α, E1β, E2, and E3. Its activity is primarily regulated by the phosphorylation/dephosphorylation of E1α [12].

Abbreviations: PDH, pyruvate dehydrogenase; PDC, pyruvate dehydrogenase complex; HIF, Hypoxia-Inducible Factor; PHD, prolyl-hydroxylase domain containing protein; MEF, mouse embryonic fibroblast; ROS, reactive oxygen species; GST, glutathione S-transferase; DFO, deferoxamine.

* Corresponding author. Address: Oxygen Biology Laboratory, Medical Research Institute, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8510, Japan. Fax: +81 3 5803 4815.
E-mail address: nakayama.mtt@mri.tmd.ac.jp (K. Nakayama).

http://dx.doi.org/10.1016/j.bbrc.2014.07.114
0006-291X/

Pyruvate dehydrogenase kinase, which is up-regulated by HIF-1, phosphorylates E1α to inhibit PDH activity and causes a metabolic shift from mitochondrial respiration to glycolysis [13,14].

2. Materials and methods

2.1. Cell culture

HeLa cells and the breast tumor MCF7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, high glucose) (Wako, Japan) containing 10% fetal bovine serum (FBS) and antibiotics. Immortalized mouse embryonic fibroblasts (MEFs) established from *PHD3*$^{+/+}$ and *PHD3*$^{-/-}$ mouse embryos were maintained in 10% FBS with 0.1 mM nonessential amino acids (Invitrogen, USA), 0.2 mM 2-mercaptoethanol (Sigma, USA) and antibiotics [15].

2.2. Hypoxic treatment

Cells were exposed to hypoxia (1% O_2, 5% CO_2, and the rest balanced with N_2) in a hypoxia workstation (Hirasawa works, Japan). An oxygen sensor was used to ensure that the oxygen concentration inside the workstation was maintained at 1% throughout the experiments (MC-8G-S, Iijima Electrics, Japan).

2.3. siRNA treatment

Three PHD3 siRNA sequences were tested, and the most effective one was selected [11]. Cells were transfected with negative control siRNA (#45-2001, Invitrogen) or PHD3 siRNA (EGLN3-HSS174178, Invitrogen) using the Lipofectamine™ RNAiMAX transfection reagent (Invitrogen). The cells were used 2 days after transfection. The efficiency of gene silencing was assessed by Western blotting.

2.4. Reagents and antibodies

The following antibodies were purchase and used: anti-β-actin, anti-Flag M2 (Sigma); anti-HIF-1α, anti-PHD3 (Novus); anti-Myc tag (Millipore); anti-PDH-E1α, anti-E1β, anti-E2, and PDH immunocapture kit (Mitoscience/Abcam). DFO was purchased from Sigma. Phos-tag acrylamide was purchased from Wako.

2.5. Immunoprecipitation and Western blotting

Cells were harvested on ice and lysed in lysis buffer (50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1 μg/ml PMSF, and 2 μg/ml leupeptin). Anti-Flag or anti-Myc antibody (1 μg) was added to the lysate, followed by the addition of protein-G agarose (Invitrogen). Mitochondrial fractions were prepared as described previously [16]. For PDC purification, PDH capture beads (20 μl) were added to the lysate. After four washes with lysis buffer, immunoprecipitates or total cell lysates were subjected to SDS–PAGE (50 μg of total lysate/lane) and then transferred onto nitrocellulose membranes (PALL, USA). *In vitro* interaction experiment was performed as described previously [11].

2.6. qPCR

Total RNA was isolated from cultured cells using the RNeasy® kit (Qiagen). First-strand cDNA synthesis was performed with 2 μg of total RNA using the PrimeScript™ II kit (Takara Bio, Japan). The cDNA synthesized was used for PCR analysis using SsoFast™ EvaGreen® Supermix (BioRad, USA) with a Chromo4™ real-time PCR detecting system (BioRad). The PDH-E1α primers were 5′-CTACCGCTACCATGGACACAGCATG-3′ (F) and 5′-CTCCTTTAATTCTTCAACACTTGCAAGA-3′ (R). The E1β primers were 5′-ATCATCTCGTGACTGTGGAAGGAGGC-3′ (F) and 5′-TTTGCGTAAGGCATAGGGACATCAGCA-3′ (R). The E2 primers were 5′-GAATCATCAAGAAGGACATTGACTCTT-3′ (F) and 5′-ATCGACAGAAAGGTAATAATGAGGTATA-3′ (R). The E3 primers were 5′-AATGTTGGCTCACAAAGCAGAAGATGA-3′ (F) and 5′-CTTCACCATGCCATCTGTGTCAGCAT-3′ (R).

2.7. Measurement of pyruvate dehydrogenase (PDH) activity

PDH activity was measured using a PDH enzyme activity dipstick assay kit (Abcam, USA) according to the manufacturer's instruction. Briefly, the active PDH complex was captured with anti-PDH antibody from the cell lysate, and its activity was measured in a colorimetric assay [17].

2.8. Measurement of oxygen consumption

PHD3$^{+/+}$ or *PHD3* $^{-/-}$ MEFs (4.0×10^4) were plated in customized 24-well plates (Seahorse Bioscience, USA) 12 h prior to the measurement. The oxygen consumption rate (OCR) was measured using an extracellular flux analyzer (Model XF24; Seahorse Bioscience).

2.9. Cell death assay and ROS measurement

Cells were plated (1.5×10^5) on a 60-mm dish and exposed to hypoxia. Cells were stained with 0.5% Trypan blue and counted. ROS production in cells was monitored using the DCFDA cellular ROS detection assay kit (Abcam).

Each experiment was performed at least three times and representative data are shown.

3. Results

3.1. Interaction of PHD3 and PDH-E1β

We first confirmed the interaction of PHD3 with PDH-E1β by exogenously expressing the proteins in 293T cells. Immunoprecipitation of PHD3 co-precipitated PDH-E1β, indicating that these proteins interact (Fig. 1A). Further, *in vitro* interaction was assessed using bacterially purified GST-PHD3 and *in vitro*-translated PDH-E1β. GST-PHD3 specifically interacted with PDH-E1β, whereas no interaction was found with GST alone (Fig. 1B). Next, the interaction between the endogenous proteins was tested in HeLa cell lysate with PDH capture beads, which immunoprecipitate the PDC. Interaction between endogenous PHD3 and PDH-E1β was detected in cells exposed to hypoxia, when PHD3 was up-regulated (Fig. 1C). Importantly, other components of the PDC (E1α and E2) were also found in the immunoprecipitate, indicating that PHD3 interacts with the whole PDC. In addition, the amount of PDH-E1α and E1β in the PDC was decreased in cells exposed to hypoxia. Of note, PDH-E3 could not be detected because no suitable antibody was available. To identify the domain of PDH-E1β required for the interaction with PHD3, we generated deletion mutants of PDH-E1β by dividing the protein into two parts without disrupting the domain structure of the protein. Results demonstrated that PHD3 interacts with the N-terminal portion of PDH-E1β (Fig. 1D). Next we compared the binding ability of the three HIF prolyl-hydroxylases, PHD1, PHD2, and PHD3. PHD3 interacted most efficiently with PDH-E1β while PHD2 interacted very weakly with it and PHD1 did not interact (Fig. 1E).

3.2. PHD3 regulates PDH activity by maintaining the PDC

Since physical interaction between PHD3 and the PDC was detected, we assessed PDH activity. Clear inhibition of PDH activity

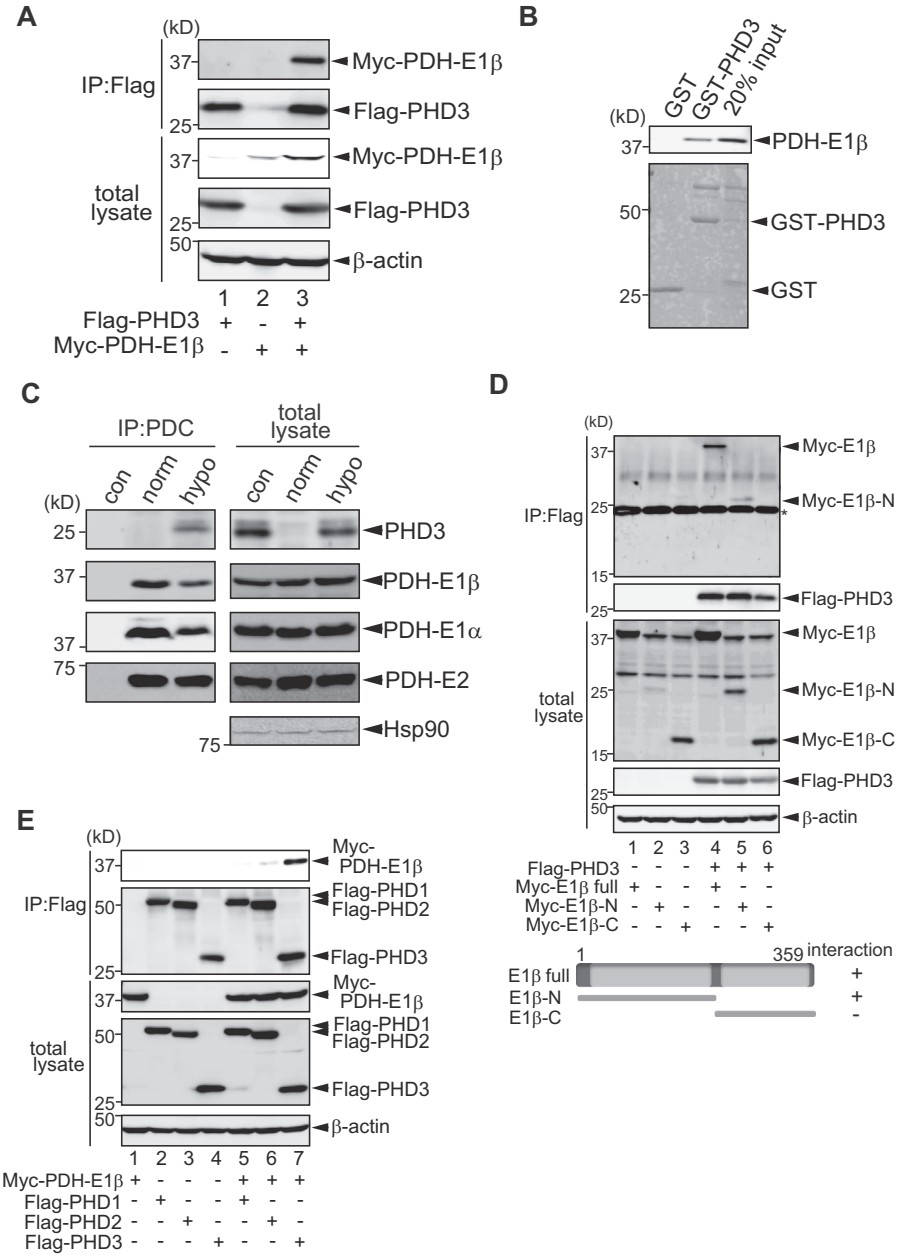

Fig. 1. Interaction of PHD3 and PDH-E1β. (A) Interaction of PHD3 and PDH-E1β in 293T cells. Myc-tagged PDH-E1β and Flag-tagged PHD3 expression vectors were co-transfected into 293T cells. Cell lysates were immunoprecipitated with anti-Flag antibody and subjected to Western blotting. Myc, Flag, and β-actin blots are shown. (B) *In vitro* interaction of PHD3 and PDH-E1β. *In vitro*-translated Myc-PDH-E1β was incubated with bacterially purified GST or GST-PHD3 fusion protein attached to glutathione beads in PBS. After incubation, the beads were extensively washed and subjected to SDS–PAGE. The association of PDH-E1β with PHD3 was detected by Western blotting. The input of GST proteins is shown as a Ponceau-S-stained membrane. (C) Interaction of endogenous PHD3 and PDH-E1β. A co-immunoprecipitation experiment was performed to assess the interaction between the endogenous proteins. HeLa cells were exposed to hypoxia for 6 h and lysed. PDH capture beads or control normal mouse IgG was added to the total lysate (10 mg) and immunoprecipitates were subjected to Western blotting. The blot was detected with anti-PHD3, PDH-E1β, E1α, E2 and Hsp90 antibodies. con: control IgG in hypoxic-treated lysate; norm: normoxia; hypo: hypoxia. (D) Interaction of PHD3 with the N-terminal region of PDH-E1β. Myc-PDH-E1β expression vectors and deletion mutants were co-transfected with Flag-PHD3 into 293T cells. Cell lysates were immunoprecipitated with anti-Flag antibody and subjected to SDS–PAGE. Myc, Flag, and β-actin blots are shown (*: IgG light chain). A schematic diagram of the PDH-E1β deletion mutants and of the interaction profile with PHD3 is shown at the bottom. (E) Interaction of PDH-E1β and PHD1, 2 and 3. A co-immunoprecipitation experiment was performed to assess the association between PDH-E1β and PHDs. Myc-tagged PDH-E1β and Flag-tagged PHD1, PHD2, or PHD3 expression vectors were co-transfected into 293T cells. Cell lysates were immunoprecipitated with anti-Flag antibody and subjected to Western blotting. Myc, Flag, and β-actin blots are shown.

was detected in cells exposed to hypoxia (Fig. 2A), which is consistent with previous reports [13]. PHD3 siRNA inhibited PDH activity in normoxia, and more remarkably and significantly in hypoxia, compared to control (Fig. 2A). Thus, we tried to characterize the mechanism underlying the decreased PDH activity caused by PHD3 knockdown by assessing the phosphorylation of the PDH-E1α subunit, the expression level of PDH subunits in mitochondria, and PDC formation. First, E1α phosphorylation was detected by electrophoresis within acrylamide gel containing phos-tag, which binds to a phosphate group of the phosphorylated protein and induces a clear mobility shift. Hypoxia clearly induced the phosphorylation of E1α in MCF7 and HeLa cells, which is consistent

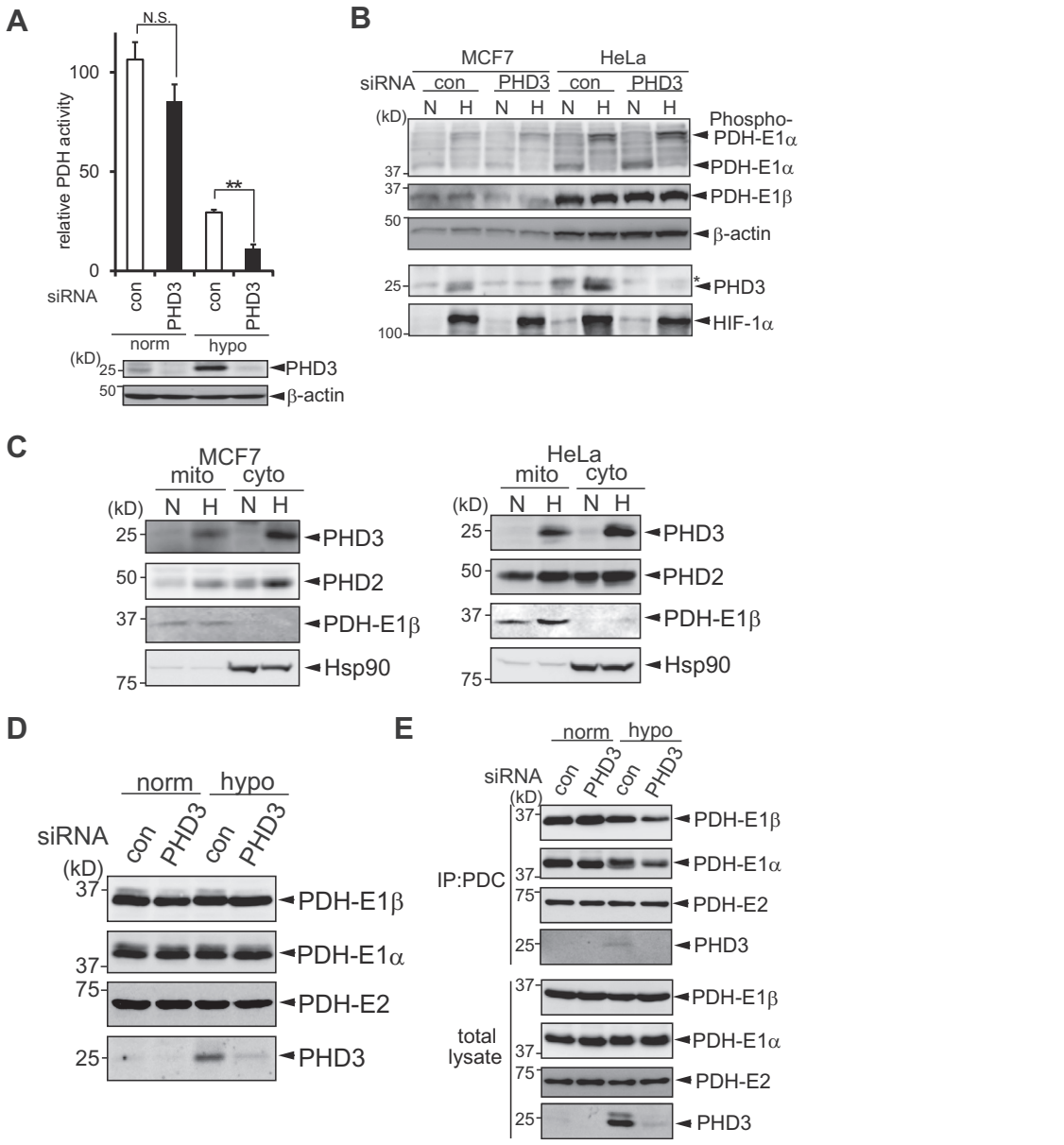

Fig. 2. PHD3-depleted cells have low levels of functional PDC and PDH activity. (A) Depletion of PHD3 by siRNA decreases PDH activity. MCF7 cells were transfected with control or PHD3 siRNA and cultured under normoxic or hypoxic conditions for 24 h. Cells were harvested and PDH activity was measured. The efficiency of the PHD3 knockdown was confirmed by Western blotting. The statistical significance of the difference was analyzed by *t* test (**$P < 0.02$; N.S.: not significant). (B) The phosphorylation status of PDH-E1α is not affected by PHD3 siRNA. MCF7 cells or HeLa cells were transfected with control or PHD3 siRNA and cultured under normoxic or hypoxic conditions for 6 h. Total cell lysate (50 μg) was subjected to phos-tag SDS–PAGE and the expression of E1α, E1β, and β-actin was detected by Western blotting. The expression of PHD3 and HIF-1α was confirmed on a regular SDS–PAGE gel followed by Western blotting. N: normoxia; H: hypoxia; *: non-specific band. (C) Mitochondrial localization of PHD3. MCF7 cells or HeLa cells were cultured under normoxic or hypoxic conditions for 6 h. Mitochondrial fractions were purified and subjected to Western blotting for PHD3, PHD2, PDH-E1β (mitochondrial protein), and Hsp90 (cytoplasmic protein). mito: mitochondrial fraction (50 μg/lane); cyto: cytoplasmic fraction (25 μg/lane). (D) The expression of PDH complex components in mitochondria is not affected by PHD3 depletion. HeLa cells were transfected with control or PHD3 siRNA and cultured under normoxic or hypoxic conditions for 6 h. Mitochondrial fractions were purified and subjected to Western blotting (50 μg/lane) with anti-E1β, E1α, E2, and PHD3 antibodies. (E) PHD3 depletion decreases the amount of PDC. HeLa cells were transfected with control or PHD3 siRNA and cultured under normoxic or hypoxic conditions for 6 h. Cell lysate was prepared and PDH capture beads were added (4.5 mg). The immunoprecipitates were subjected to Western blotting. The blot was detected with anti-E1β, E1α, E2, and PHD3 antibodies.

with previous results [13]. Yet, a comparable level of phosphorylation was observed in cells transfected with control and PHD3 siRNA, indicating that E1α phosphorylation is not the cause of the reduced PDH activity (Fig. 2B). PDC mainly localizes and functions in the mitochondria. PHD3 was reported to localize in the nucleus and cytoplasm [18], but it remains unknown if it localizes in the mitochondria. Western blotting of mitochondrial fractions derived from MCF7 and HeLa cells demonstrated the existence of PHD3 in mitochondria (Fig. 2C). PHD2 was also found in the mitochondria as reported [19]. Thus, we examined the amount of each PDH subunit in mitochondria, to assess potential changes upon PHD3 knockdown. The amount of PDH-E1β, E1α, and E2 were unaffected by PHD3 knockdown in both normoxic and hypoxic conditions (Fig. 2D). Thirdly, the PDC was quantified by immunopurification. Western blotting confirmed that hypoxia reduced PDH-E1β and E1α in the PDC, and that both were further reduced in cells treated

with PHD3 siRNA (Fig. 2E). Since the PDC capture beads capture PDH-E2, no change was detected in the amount of PDH-E2 in the complex.

3.3. *PHD3*$^{-/-}$ MEFs have decreased PDH activity and are resistant to cell death during prolonged hypoxia

We next used *PHD3*$^{+/+}$ and *PHD3*$^{-/-}$ MEFs to further characterize the role of PHD3 in regulating PDH activity. Immunopurification of PDC revealed that *PHD3*$^{-/-}$ cells have significantly less PDC than *PHD3*$^{+/+}$ cells, whereas the levels of PDH subunits expressed in the cells are comparable (Fig. 3A). Accordingly, *PHD3*$^{-/-}$ cells have less PDH activity in normoxia, and the difference was more obvious in hypoxia (Fig. 3B). The expression levels of *PDH-E1α*, *E1β*, *E2*, and *E3* were comparable between *PHD3*$^{+/+}$ and *PHD3*$^{-/-}$ MEFs at the mRNA level (Fig. 3C). Altogether, these results indicate that *PHD3*$^{-/-}$ cells have less functional PDC, thus less enzymatic activity. In accordance with this, *PHD3*$^{-/-}$ cells consume less oxygen than *PHD3*$^{+/+}$ cells (Fig. 3D), since low PDH activity would limit mitochondrial respiration. To identify any phenotypic change in

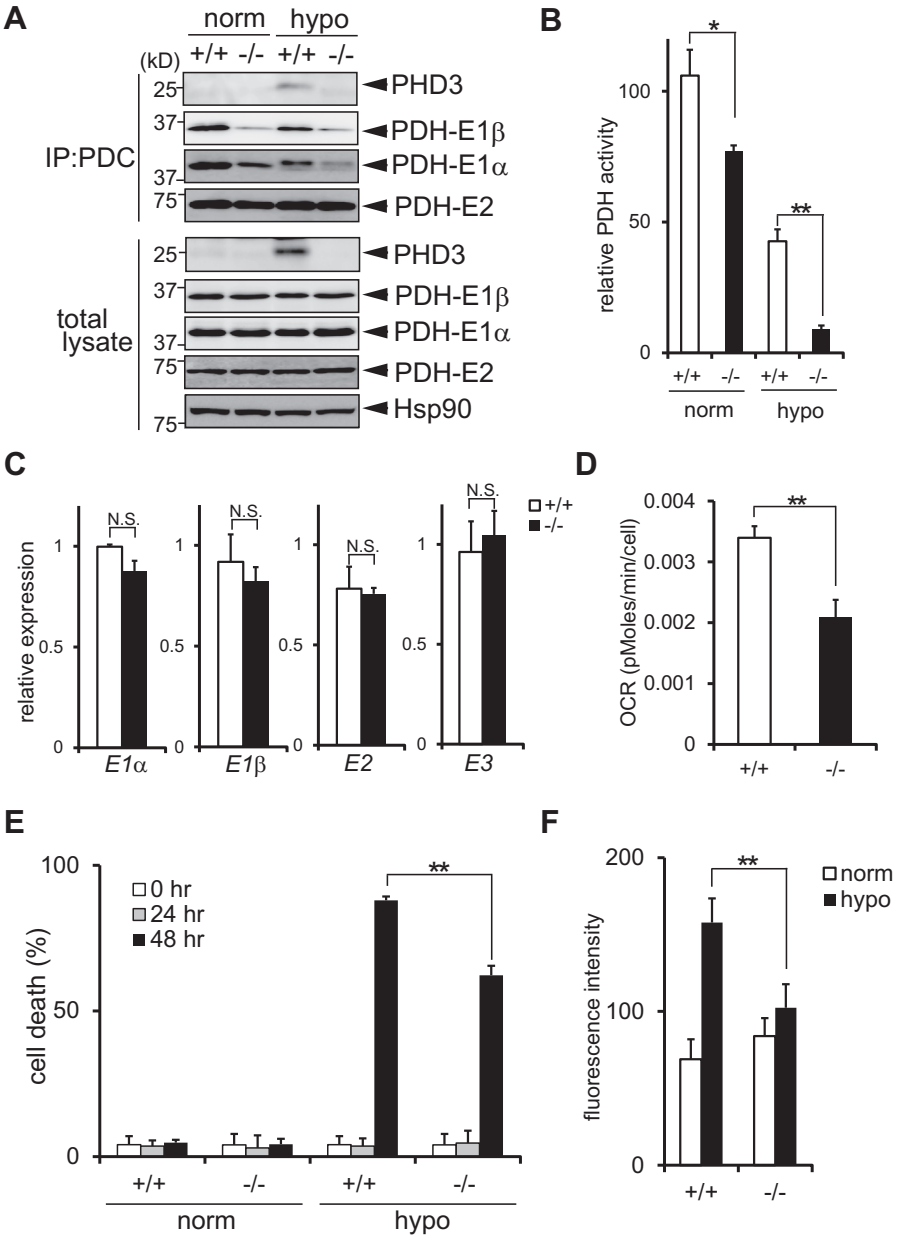

Fig. 3. *PHD3*$^{-/-}$ MEFs exhibit decreased PDH activity and are resistant to cell death during prolonged hypoxia. (A) *PHD3*$^{-/-}$ MEFs have reduced levels of functional PDC. *PHD3*$^{+/+}$ and *PHD3*$^{-/-}$ MEFs were cultured under normoxic or hypoxic conditions for 6 h. Cell lysate was prepared and PDH capture beads were added (3.5 mg). The immunoprecipitates were subjected to Western blotting and detected with anti-PHD3, PDH-E1β, E1α, E2 and Hsp90 antibodies. (B) Reduced PDH activity in *PHD3*$^{-/-}$ MEFs. *PHD3*$^{+/+}$ and *PHD3*$^{-/-}$ MEFs were cultured under normoxic or hypoxic conditions for 24 h. The cells were harvested and PDH activity was measured. (C) Comparable expression of PDH subunits in *PHD3*$^{+/+}$ and *PHD3*$^{-/-}$ MEFs. The mRNA expression level of *PDH-E1α*, *E1β*, *E2*, and *E3* (normalized against β-actin) were compared by qPCR in *PHD3*$^{+/+}$ and *PHD3*$^{-/-}$ MEFs. (D) Low oxygen consumption rate in *PHD3*$^{-/-}$ MEFs. *PHD3*$^{+/+}$ and *PHD3*$^{-/-}$ MEFs were plated in 24-well plates 12 h prior to the measurement. The oxygen consumption rate (OCR) was measured using an extracellular flux analyzer. (E, F) *PHD3*$^{-/-}$ MEFs produce less ROS and are more resistant to cell death during prolonged hypoxia than control cells. *PHD3*$^{+/+}$ and *PHD3*$^{-/-}$ MEFs cultured in low-glucose medium (1.0 g/L) were exposed to hypoxia. The population of cells undergoing cell death was measured by Trypan blue exclusion (E). ROS were measured after 24 h in normoxia or hypoxia using the ROS detection kit (F). The error bars indicate SEMs. The statistical significance of the difference was analyzed by *t* test (*$P < 0.05$; **$P < 0.02$; N.S.: not significant).

PHD3$^{-/-}$ cells, cells were cultured in low-glucose medium and exposed to hypoxia for 48 h. As a result, around 90% cell death was observed in *PHD3*$^{+/+}$ cells at 48 h, while only about 60% cell death was measured in *PHD3*$^{-/-}$ cells (Fig. 3E). Further, ROS production was found to be higher in *PHD3*$^{+/+}$ cells compared to *PHD3*$^{-/-}$ cells during prolonged hypoxia (Fig. 3F). This is possibly due to the higher activity of PDH in *PHD3*$^{+/+}$ cells, which leads to more respiration and ROS production in mitochondria in hypoxia, thus causing greater cell death.

3.4. The prolyl-hydroxylase activity of PHD3 is not necessary for PDC formation

Finally, we examined whether PHD3 prolyl-hydroxylase activity is required for PDC formation. Since PHD3 enzymatic activity requires Fe^{2+} as a co-factor, we used the iron-chelating reagent deferoxamine (DFO) to inhibit PHD3 activity. Treatment with DFO for 16 h, which is sufficient to inhibit PHD activity, did not alter PDC formation (Fig. 4A) or inhibit PDH activity (Fig. 4B). These results indicate that PHD3 enzymatic activity is not necessary for PDC formation or PDH activity. To further support this, the effect of DFO on the PDH-E1β and PHD3 interaction was examined by co-immunoprecipitation in 293T cells. PHD3 was found to associate equally with PDH-E1β in the presence or absence of DFO (Fig. 4C). Moreover, the catalytically inactive PHD3 mutants H196R and R205K associated with PDH-E1β similarly (Fig. 4D). Taken together, these results suggest that PHD3 activity or hydroxylation of PDH subunits is not necessary for PDH activity.

4. Discussion

The present study shows that functional PDC decreases in hypoxia, and decreases further in *PHD3*$^{-/-}$ cells or when PHD3 is depleted by siRNA (Figs. 1C, 2E, and 3A). This indicates that PHD3 has a positive role in the maintenance of the PDC. Decrease in the amount of PDH-E1α/E1β in PDC might constitute another layer of PDH regulation in hypoxia, in addition to phosphorylation, to ensure the inhibition of PDH activity. By contrast, it would be beneficial for cells to maintain PDH activity for efficient ATP production, if a certain amount of oxygen remains available for oxidative phosphorylation, as in moderate hypoxia. Several reports demonstrated that PHD3 forms a complex by interacting with multiple proteins, and possibly serves as a molecular scaffold [10,20,21]. Thus, PHD3 may function as a scaffold in hypoxia to

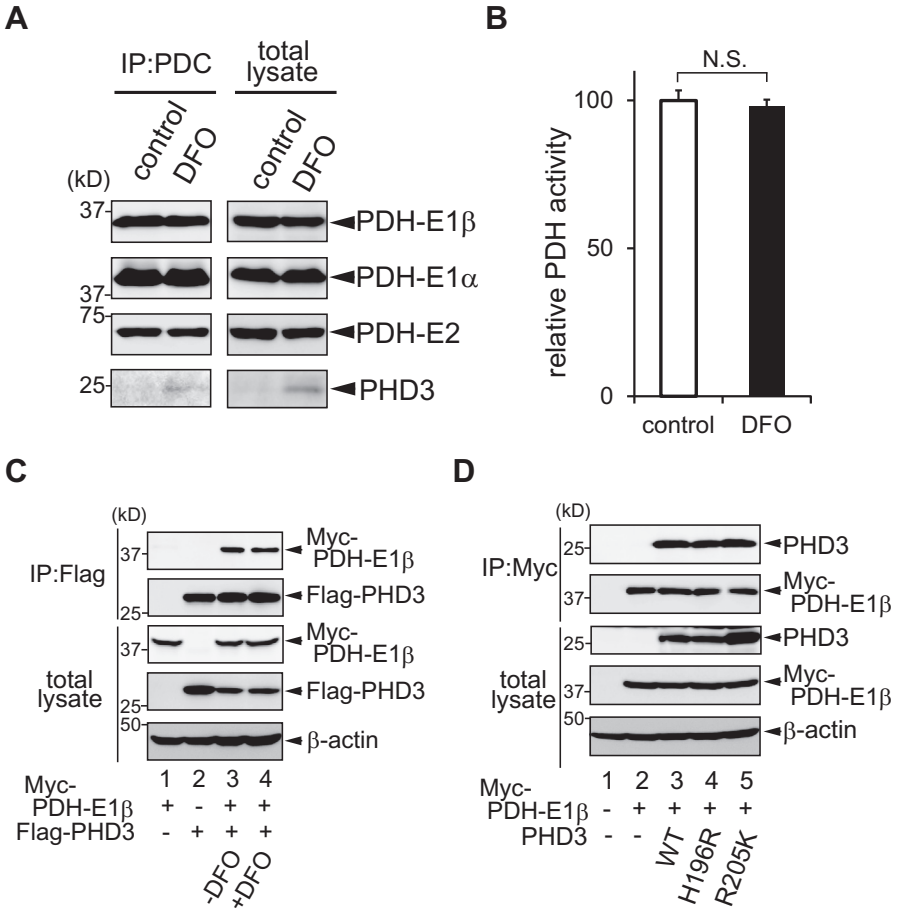

Fig. 4. The enzymatic activity of PHD3 is not necessary for the regulation of PDH activity. (A) PDC formation is not affected by the prolyl-hydroxylase inhibitor DFO. HeLa cells were exposed to DFO (10 μM) for 16 h under normoxic conditions. Cell lysate was prepared and PDH capture beads were added (5.5 mg). The immunoprecipitates were subjected to Western blotting and detected with anti-E1β, E1α, E2, and PHD3 antibodies. (B) The PDH activity is not affected by DFO. MCF7 cells were exposed to DFO (10 μM) for 16 h under normoxic conditions. Cells were harvested and PDH activity was measured. The statistical significance of the difference was analyzed by *t* test (N.S.: not significant). (C) The interaction of PDH-E1β with PHD3 is not affected by DFO. A co-immunoprecipitation experiment was performed in 293T cells co-transfected with Myc-PDH-E1β and Flag-PHD3 exposed to DFO (10 μM). Cell lysates were immunoprecipitated with anti-Flag antibody and subjected to Western blotting. Myc, Flag, and β-actin blots are shown. (D) Enzymatically inactive PHD3 mutants interact with PDH-E1β in a manner similar to that of wild-type PHD3. A co-immunoprecipitation experiment was performed in 293T cells co-transfected with Myc-PDH-E1β and wild-type PHD3 (no tag) or the enzymatically inactive mutants H196R or R205K. The expression of PHD3, PDH-E1β and β-actin was detected.

fine-tune PDH activity. Importantly, decreased PDH activity was also found in PHD3-depleted cells and in *PHD3*$^{-/-}$ cells in normoxia. This suggests that PHD3 maintains steady-state levels of the PDC. The mechanism by which PHD3 is involved in PDC assembly or turnover will be identified in future studies.

Less cell death is observed in the brain of *PHD3*$^{-/-}$ mice and in neuronal cells derived from *PHD3*$^{-/-}$ animals than in controls [15,22]. The c-jun/PHD3/KIF1Bbeta pathway plays a critical role in such neuronal cell death [15]. Our study also demonstrated that *PHD3*$^{-/-}$ cells produce less ROS and are more resistant to cell death during prolonged hypoxia than *PHD3*$^{+/+}$ cells (Fig. 3E and F). PHD3 may positively regulate PDH activity to maintain ATP levels in the early phase of hypoxia, but may end up having negative effects on the cell if hypoxia is sustained. Although it is not clear whether there is any interplay between the PDH regulation and the c-jun/PHD3/KIF1Bbeta pathway, PHD3 might potentially function to induce cell death in cells.

Decreased PDH activity leads to the neuronal disorder Leigh syndrome [23]. *PHD3*$^{-/-}$ cells also have decreased functional PDC and PDH activity. Typical symptoms of Leigh syndrome are hypo/hypertonia or peripheral neuropathy [23], which are also observed in *PHD3*$^{-/-}$ mice [22]. Thus, further analysis of *PHD3*$^{-/-}$ mice would clarify the pathophysiological importance of the present study.

Acknowledgments

We thank Dr. Hiroshi Nishina and members of his laboratory for helpful discussions during the course of the study. We also thank Dr. Noriyo Hayakawa and Ms. Makiko Nawa for technical assistance. K.N. was supported by the Takeda Science Foundation, the Tokyo Biochemical Research Foundation and the Kato Memorial Bioscience Foundation. This study was also supported by a Grant-in-Aid for Scientific Research C (JSPS).

References

[1] W.G. Kaelin Jr., P.J. Ratcliffe, Oxygen sensing by metazoans: the central role of the HIF hydroxylase pathway, Mol. Cell 30 (2008) 393–402.
[2] G.L. Semenza, Targeting HIF-1 for cancer therapy, Nat. Rev. Cancer 3 (2003) 721–732.
[3] A.C. Epstein, J.M. Gleadle, L.A. McNeill, K.S. Hewitson, J. O'Rourke, D.R. Mole, M. Mukherji, E. Metzen, M.I. Wilson, A. Dhanda, Y.M. Tian, N. Masson, D.L. Hamilton, P. Jaakkola, R. Barstead, J. Hodgkin, P.H. Maxwell, C.W. Pugh, C.J. Schofield, P.J. Ratcliffe, *C. elegans* EGL-9 and mammalian homologs define a family of dioxygenases that regulate HIF by prolyl hydroxylation, Cell 107 (2001) 43–54.
[4] P. Koivunen, P. Tiainen, J. Hyvarinen, K.E. Williams, R. Sormunen, S.J. Klaus, K.I. Kivirikko, J. Myllyharju, An endoplasmic reticulum transmembrane prolyl 4-hydroxylase is induced by hypoxia and acts on hypoxia-inducible factor alpha, J. Biol. Chem. 282 (2007) 30544–30552.
[5] E. Berra, E. Benizri, A. Ginouves, V. Volmat, D. Roux, J. Pouyssegur, HIF prolyl-hydroxylase 2 is the key oxygen sensor setting low steady-state levels of HIF-1alpha in normoxia, EMBO J. 22 (2003) 4082–4090.
[6] R.J. Appelhoff, Y.M. Tian, R.R. Raval, H. Turley, A.L. Harris, C.W. Pugh, P.J. Ratcliffe, J.M. Gleadle, Differential function of the prolyl hydroxylases PHD1, PHD2, and PHD3 in the regulation of hypoxia-inducible factor, J. Biol. Chem. 279 (2004) 38458–38465.
[7] E.P. Cummins, E. Berra, K.M. Comerford, A. Ginouves, K.T. Fitzgerald, F. Seeballuck, C. Godson, J.E. Nielsen, P. Moynagh, J. Pouyssegur, C.T. Taylor, Prolyl hydroxylase-1 negatively regulates IkappaB kinase-beta, giving insight into hypoxia-induced NFkappaB activity, Proc. Natl. Acad. Sci. U.S.A. 103 (2006) 18154–18159.
[8] W. Luo, H. Hu, R. Chang, J. Zhong, M. Knabel, R. O'Meally, R.N. Cole, A. Pandey, G.L. Semenza, Pyruvate kinase M2 is a PHD3-stimulated coactivator for hypoxia-inducible factor 1, Cell 145 (2011) 732–744.
[9] O. Mikhaylova, M.L. Ignacak, T.J. Barankiewicz, S.V. Harbaugh, Y. Yi, P.H. Maxwell, M. Schneider, K. Van Geyte, P. Carmeliet, M.P. Revelo, M. Wyder, K.D. Greis, J. Meller, M.F. Czyzyk-Krzeska, The von Hippel–Lindau tumor suppressor protein and Egl-9-type proline hydroxylases regulate the large subunit of RNA polymerase II in response to oxidative stress, Mol. Cell. Biol. 28 (2008) 2701–2717.
[10] K. Nakayama, S. Gazdoiu, R. Abraham, Z.Q. Pan, Z. Ronai, Hypoxia-induced assembly of prolyl hydroxylase PHD3 into complexes: implications for its activity and susceptibility for degradation by the E3 ligase Siah2, Biochem. J. 401 (2007) 217–226.
[11] M. Sato, M. Sakota, K. Nakayama, Human PRP19 interacts with prolyl-hydroxylase PHD3 and inhibits cell death in hypoxia, Exp. Cell Res. 316 (2010) 2871–2882.
[12] M.S. Patel, L.G. Korotchkina, Regulation of the pyruvate dehydrogenase complex, Biochem. Soc. Trans. 34 (2006) 217–222.
[13] J.W. Kim, I. Tchernyshyov, G.L. Semenza, C.V. Dang, HIF-1-mediated expression of pyruvate dehydrogenase kinase: a metabolic switch required for cellular adaptation to hypoxia, Cell Metab. 3 (2006) 177–185.
[14] I. Papandreou, R.A. Cairns, L. Fontana, A.L. Lim, N.C. Denko, HIF-1 mediates adaptation to hypoxia by actively downregulating mitochondrial oxygen consumption, Cell Metab. 3 (2006) 187–197.
[15] S. Schlisio, R.S. Kenchappa, L.C. Vredeveld, R.E. George, R. Stewart, H. Greulich, K. Shahriari, N.V. Nguyen, P. Pigny, P.L. Dahia, S.L. Pomeroy, J.M. Maris, A.T. Look, M. Meyerson, D.S. Peeper, B.D. Carter, W.G. Kaelin Jr., The kinesin KIF1Bbeta acts downstream from EglN3 to induce apoptosis and is a potential 1p36 tumor suppressor, Genes Dev. 22 (2008) 884–893.
[16] N. Ishihara, Y. Eura, K. Mihara, Mitofusin 1 and 2 play distinct roles in mitochondrial fusion reactions via GTPase activity, J. Cell Sci. 117 (2004) 6535–6546.
[17] S. Kamarajugadda, L. Stemboroski, Q. Cai, N.E. Simpson, S. Nayak, M. Tan, J. Lu, Glucose oxidation modulates anoikis and tumor metastasis, Mol. Cell. Biol. 32 (2012) 1893–1907.
[18] E. Metzen, U. Berchner-Pfannschmidt, P. Stengel, J.H. Marxsen, I. Stolze, M. Klinger, W.Q. Huang, C. Wotzlaw, T. Hellwig-Burgel, W. Jelkmann, H. Acker, J. Fandrey, Intracellular localisation of human HIF-1 alpha hydroxylases: implications for oxygen sensing, J. Cell Sci. 116 (2003) 1319–1326.
[19] S. Barth, F. Edlich, U. Berchner-Pfannschmidt, S. Gneuss, G. Jahreis, P.A. Hasgall, J. Fandrey, R.H. Wenger, G. Camenisch, Hypoxia-inducible factor prolyl-4-hydroxylase PHD2 protein abundance depends on integral membrane anchoring of FKBP38, J. Biol. Chem. 284 (2009) 23046–23058.
[20] N. Masson, R.J. Appelhoff, J.R. Tuckerman, Y.M. Tian, H. Demol, M. Puype, J. Vandekerckhove, P.J. Ratcliffe, C.W. Pugh, The HIF prolyl hydroxylase PHD3 is a potential substrate of the TRiC chaperonin, FEBS Lett. 570 (2004) 166–170.
[21] K. Rantanen, J. Pursiheimo, H. Hogel, V. Himanen, E. Metzen, P.M. Jaakkola, Prolyl hydroxylase PHD3 activates oxygen-dependent protein aggregation, Mol. Biol. Cell 19 (2008) 2231–2240.
[22] T. Bishop, D. Gallagher, A. Pascual, C.A. Lygate, J.P. de Bono, L.G. Nicholls, P. Ortega-Saenz, H. Oster, B. Wijeyekoon, A.I. Sutherland, A. Grosfeld, J. Aragones, M. Schneider, K. van Geyte, D. Teixeira, A. Diez-Juan, J. Lopez-Barneo, K.M. Channon, P.H. Maxwell, C.W. Pugh, A.M. Davies, P. Carmeliet, P.J. Ratcliffe, Abnormal sympathoadrenal development and systemic hypotension in PHD3$^{-/-}$ mice, Mol. Cell. Biol. 28 (2008) 3386–3400.
[23] K.P. Patel, T.W. O'Brien, S.H. Subramony, J. Shuster, P.W. Stacpoole, The spectrum of pyruvate dehydrogenase complex deficiency: clinical, biochemical and genetic features in 371 patients, Mol. Genet. Metab. 106 (2012) 385–394.